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(54) Title: SOLID MEDIUM AND METHOD FOR DNA STORAGE			
(57) Abstract A solid medium for storage of DNA, including blood DNA, comprising a solid matrix having a compound or composition which protects against degradation of DNA incorporated into or absorbed on the matrix. Methods for storage of DNA using this solid medium, and for recovery of DNA or <i>in situ</i> use of DNA are also disclosed.			

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SOLID MEDIUM AND METHOD FOR DNA STORAGE

This invention relates to a solid medium for use in the storage of DNA, particularly DNA in blood samples, and to methods which comprise the use of this solid medium. In particular, the invention relates to a method for storage and transport of DNA on the solid medium, as well as to methods which involve either (a) the recovery of the DNA from the solid medium or (b) the use of the DNA in situ on the solid medium (for example, DNA sequence amplification by the polymerase chain reaction).

The DNA in blood samples (hereinafter referred to as "blood DNA") is used for the purposes of diagnosis of genetic diseases, diagnosis and monitoring of blood-borne parasitic diseases such as malaria, the determination of paternity, and the monitoring of other unusual cell populations in the blood as can occur in some neoplasias. For these purposes, the term "blood DNA" is used to cover all sources of DNA commonly found in blood, and thus includes the DNA of the human patient from whom the blood sample was obtained, as well as the DNA in any other organisms circulating within his/her blood.

It is a principal object of the present invention to provide a solid medium and method whereby DNA, particularly DNA in blood samples, may be conveniently and reliably stored, and if desired transported (for

example from a hospital or doctor's surgery to a pathology laboratory), in a form suitable for either the recovery of the DNA for analysis or the use of the DNA in situ on the solid medium.

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In the past, blood DNA has been transported as purified DNA, liquid blood, frozen blood or blood dried onto paper, however, all these methods have disadvantages. Transport of blood DNA as dried, purified DNA is most desirable, but it requires a high standard of technical assistance to be available at the places of collection. Thus, since such technical assistance is usually not available at the collection places, whole blood or other crude samples are usually sent to a central facility where the DNA is purified. Transport of liquid blood involves the need for sterility of collection and this is extremely inconvenient under some circumstances, e.g. where the sample is a heel-prick taken from an infant. The transport of liquid blood or frozen blood also demands temperature control and an appropriate transport system other than the regular postal system. This is true even before the question of hygiene is considered. In addition, the modern problems with pathogens such as the "AIDS" virus completely rule out the transport of any potentially infective liquid or frozen sample except under proper and expensive supervision.

Blood dried onto filter paper is a proven alternative to the above procedures and it has been shown that DNA can be extracted and isolated from dried whole blood spots in a form and in sufficient quantities for use in DNA analysis¹. This procedure still suffers from a number of disadvantages. Thus, there has been no deliberate and rapid destruction of most pathogens, and there has been no deliberate inhibition of the processes degrading the DNA other than that caused by desiccation.

Slow desiccation or even a small degree of rehydration under conditions of high relative humidity will allow the growth of DNA-destroying microflora. Even in the presence of bacteriostatic agents of the type that do not denature proteins, there will be conditions that permit enzymic-autolytic breakdown of the DNA and some nonenzymic breakdown of the DNA (in enzymic-autolytic breakdown, dying or damaged tissues, either human cells or parasite cells, activate enzymes that degrade their own components). There is also considerable difficulty desorbing very high molecular weight DNA from paper, if this is required. Surface adsorption effects can cause losses of DNA and this will cause the preferential loss of the least degraded, i.e. the most desired class of DNA molecules.

According to the present invention there is provided a solid medium for storage of DNA, including blood DNA, which comprises a solid matrix having a compound or composition which protects against degradation of DNA incorporated into or absorbed on the matrix.

Preferably, the solid matrix comprises a solid support, for example, an absorbent cellulose-based paper (such as filter paper) or a micromesh of synthetic plastics material, with the DNA-protecting compound or composition absorbed onto the solid support. Alternatively, the solid matrix may include a suitable binder material so that the matrix is in the form of a compressed tablet or pellet, with the DNA-protecting compound or composition incorporated into or absorbed onto the tablet or pellet.

In its broadest aspect, the present invention also provides a method for the storage of DNA, including blood DNA, which comprises applying the DNA to a solid medium

which comprises a solid matrix having a compound or composition which protects against degradation of DNA incorporated into or adsorbed on the matrix.

5 In one embodiment of the invention, particularly for long term storage of purified DNA, the DNA-protecting compound or composition comprises uric acid, together with a weak base to convert the uric acid to a urate salt and to provide an alkaline pH between 8.0 and 9.5.

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 In one particularly preferred aspect of the present invention, there is provided a solid medium for storage of blood DNA, which comprises a solid matrix having incorporated therein or absorbed thereon a
15 composition comprising a weak base, a chelating agent and an anionic surfactant or detergent, and optionally uric acid or a urate salt.

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 Preferably, the composition imposes an alkaline pH, such as a pH of between 8.0 and 9.5, on blood that is added to the matrix.

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 A further aspect of the present invention is the long term storage of DNA on the solid medium of this invention, by impregnating the solid medium or encasing the solid medium in a protective material, for example a plastics material such as polystyrene, which can be subsequently removed when access to the stored DNA is required.

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 In practice, in the storage of Blood DNA the blood sample is applied as a blood spot to the solid medium of this aspect of the invention, where the components more particularly the surfactant will denature proteins and
35 the majority of any pathogenic organisms in the sample. At the same time, however, the blood DNA will be protected from degradation factors and processes of the

type described above so that the relatively stable, and denatured, dried blood sample can then be transported to a diagnostic laboratory. There, the DNA can be extracted or the DNA used in situ on the solid medium.

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Preferably, the composition used in this aspect of this invention comprises the following:

- (i) a monovalent weak base (such as "Tris", tris-hydroxymethyl methane, either as the free base or as the carbonate);
- (ii) a chelating agent (such as EDTA, ethylene diamine tetracetic acid); and
- (iii) an anionic detergent (such as SDS, sodium dodecyl sulphate); and optionally
- (iv) uric acid or a urate salt.

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By way of example, a particularly preferred solid medium according to this aspect of the invention comprises an absorbent cellulose-based paper such as filter paper having a minimal loading, per sq.cm. of paper, as follows:

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- (a) EDTA: 0.5 micromols (146.1 mg of free acid)
- (b) Tris: 8 micromols (968.8 mg of free base)
- (c) SDS: 1 mg; and optionally
- (d) uric acid: 2 micromols (336.24 mg of acid).

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Although not vital for the short-term storage of DNA on the solid medium, the use of uric acid or a urate salt in accordance with this invention has been found to be particularly important for the long term storage of DNA as this component performs a number of functions. Firstly, it is converted into allantoin in acting as a "free-radical" trap that preferentially accepts free radicals, that would otherwise damage the base guanine in the DNA, (e.g. ^{2,3}). Such free radicals are generated by the spontaneous oxidation of thio groups in the denatured

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serum protein, and may also be generated by the large amount of iron in blood⁴. The uric acid also acts as a component of the buffering system in that it is a weak acid. It also acts as an erodible surface in that it is sparingly soluble so that DNA-containing material dried onto its crystals will be released as the urate beneath them erodes.

As previously described, the composition may include a base, optionally a monovalent weak base, to cause an alkaline pH between 8.0 and 9.5 to be imposed upon the blood that is placed upon the matrix. This is to ensure the proper action of the chelating agent in binding divalent metals. It is also to prevent the action of acid nucleases that are not so dependent on divalent metals. The base may be a weak organic base, such as Tris. Alternatively, an inorganic base such as an alkali metal carbonate or bicarbonate, for example sodium, lithium or potassium carbonate or bicarbonate, may be used.

The chelating agent is preferably a strong chelating agent such as EDTA, however a wide range of suitable strong chelating agents are commercially available. The function of the chelating agent is to bind the divalent metal ions, magnesium and calcium, and also to bind transition metal ions, particularly iron. Both calcium and magnesium are known to promote DNA degradation by acting as co-factors for enzymes. The metal ions such as iron, that readily undergo oxidation and reduction also damage nucleic acids by the production of free radicals⁴.

The anionic surfactant or detergent is included in the composition of this aspect of the invention as the primary denaturing agent. Any strong anionic detergent that binds to and denatures proteins is suitable, and as

well as SDS mentioned above other detergents such as sodium lauryl sarcosinate may also be used. This anionic detergent causes most pathogens to be inactivated due to the non-specific destruction of the secondary structure of their coat proteins, their internal proteins, and any membranes they may be dependent upon. There are exceptions, since the anionic detergent does not inactivate the most resistant bacterial spores, nor does it inactivate some extremely stable enteric virions. However, these exceptions are agents that are already likely to be transferred by ordinary contact and there is currently no great concern that these agents constitute a risk from blood.

In tests leading to the present invention, it has been demonstrated that blood DNA can be extracted satisfactorily from detergent-treated paper and that uric acid salt protects purified DNA against degradation during storage for more than 36 months. In further tests, DNA on filter paper specially treated in accordance with this invention was purified in situ, then subjected to the polymerase chain reaction (PCR). Details of these tests are set out in the following Examples.

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EXAMPLE 1. Collection and Extraction of DNA:

Sodium dodecyl sulphate was applied to Whatman 3mm paper in a solution such that there was approximately 50 μ l per sq.cm. of a solution of 2% sodium dodecyl sulphate, 10mM EDTA, and 60mM tris (free base), i.e. approximately 1 mg of sodium dodecyl sulphate per sq.cm. The paper was then dried.

The treated paper was soaked with drops of blood from various primates. The blood-stained paper was dried, sent through the ordinary mail so that it spent at least three days in the mail, and then had the DNA

extracted from it using standard procedures involving detergent-aided proteolysis and phenol extraction of the paper. The resultant DNA was then tested for its quality by being digested with restriction endonucleases and the
5 fragments analyzed by agarose gel electrophoresis.

The DNA fragments were found to be as high in quality as DNA produced from fresh blood. This demonstrates that the DNA can be extracted from
10 detergent-treated papers and that the DNA is of sufficient quality for most normal purposes.

EXAMPLE 2. Long-term Storage of Semi-purified or Purified DNA:

15 Storage of DNA, such as plasmids or other viral replicating forms, has been carried out using record cards made of absorbent paper previously soaked in a solution of uric acid and tris (free base). The cards are subsequently plasticised for further protection.
20 This procedure has been established for the long-term storage of clones from massed dot-blotting when it is possible that the original material is required at some much later date, and when a great many such massed clonings are to be kept in orderly, low-volume, files.
25 Samples have been stored successfully this way for about four years.

2.1 Preparation of DNA Record Cards

Record cards can be prepared in batches and stored
30 until needed. Whatman No.1 paper about 10cm x 15cm in size and with appropriate places marked out with an "indian ink" (i.e. colloidal carbon ink-stamp) is suitable, and any special notes on the cards can be made with an ordinary "lead" pencil (i.e. Graphite pencil).
35 Preferably, the cards are marked out in a regular pattern to assist in systematic storage and retrieval of DNA samples.

Marked cards are wrapped in clean paper, then foil, and autoclaved with a dry cycle. They are then treated with a solution of 40mM uric acid and 100mm tris (free base). The function of the urate is to protect the DNA from aging and to aid the desorption from the paper if required. These treated record cards can then be kept until required.

10 2.2 DNA samples and their application.

DNA to be stored is taken up in a dilute alkaline buffer containing EDTA, e.g. TE buffer (Tris-EDTA pH 8.0). By way of example, approx 1 ml of bacterial culture containing plasmid, is treated by the alkaline lysis method, with one phenol extraction and one alcohol precipitation, to get approx 50 μ l of plasmid or other DNA in TE buffer. A 5 μ l aliquot of each DNA sample is used to make a spot on the urate treated record card.

20 2.3 Impregnation of DNA-loaded cards with plastic.

Once a card is fully loaded with DNA samples, the DNA spots are thoroughly air-dried, then the card is dipped in 5ml of 12% w/v polystyrene in acid-free chloroform. This is preferably achieved by putting the card in a fitting polyethylene baglet and then pouring the polystyrene solution into it, spreading the polystyrene solution to thoroughly coat the card and then stripping off the soiled polyethylene. The card is then allowed to dry at room temperature.

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2.4 Storage Conditions.

The cards are conveniently stored in a sealed container in a refrigerator freezer (about -15°C) in the presence of drying agent such as silica gel and a few grains of dry sodium carbonate to remove any traces of acid vapours.

2.5 Using DNA on plasticised cards.

The storage container is allowed to rise to room temperature in order to minimise unnecessary wetting and drying cycles on long-term storage cards. The appropriate card is abstracted, the relevant DNA spot identified and a small sample of it cut out.

Since the 5 μ l spots are laid out in a regular fashion, it is quite practical to cut them across with a scalpel blade to remove a portion of the sample, e.g. one-quarter of a spot. This is then placed in a disposable plastic tube with approximately 5 ml of acid-free chloroform, capped and rotated on a blood rotator at room temperature for at least 30 minutes. This removes the protective polystyrene and effectively sterilises the sample also. DNA may then be eluted from the sample or the sample can be treated and used in situ, for example in a DNA sequence amplification reaction (PCR) (see later).

In an example, the desorption of DNA samples, both single and double stranded, from Whatman No.1 paper soaked with a solution of 40mM uric acid, and 100mM tris (free base) was examined by using the plasmid pUC19 as a source of standard double stranded DNA and M13 as a source of single stranded DNA.

In both cases, samples of the DNA were dried onto paper from solutions in TE buffer and the paper was then sheathed in protective polystyrene, as described above. The paper was later chloroform extracted to remove the protective layers, the DNA extracted with fresh TE buffer and the efficiency of extraction estimated by observing the transformation of the E.coli strain JM101 by the extracted DNA. This procedure was carried out with both untreated paper and paper that had been pre-soaked and dried with the above urate solution. Much less than 10%

of transformationally active DNA was successfully recovered from the untreated paper, whereas the treated paper gave recoveries of approximately 100%.

5 Further DNA samples which had been applied to treated paper and sheathed as previously described, were tested after 36 months storage in a dry atmosphere at - 15°C. The samples again had the plastic stripped off with chloroform, the DNA (only pUC19) recovered by simple
10 extraction with TE buffer, and the DNA activity tested as previously described. Again the activity of the DNA was high, compatible with virtually no loss of activity.

EXAMPLE 3. In situ Use of Stored Blood DNA in PCR.

15 Blood DNA stored on filter paper treated in accordance with the present invention can be amplified in situ by the polymerase chain reaction (PCR) technique. The treated paper used in this Example was Whatman 3 mm paper treated with a solution comprising, per sq.cm of
20 paper, 2 micromols uric acid, 8 micromols tris (free base), 0.5 micromols EDTA and 1 mg SDS. The stored blood DNA was treated to remove protein, then washed to remove phenol and add suitable ions, prior to DNA amplification.

25 **3.1 Solutions**

Solution A:

One-phase Phenol solution. A suitable mixture is phenol, 50 gm containing 120 mg of 8-hydroxyquinoline that has been saturated with 10
30 ml of 1.0 M tris-acetate pH 8.0 and 1.0 ml 2-mercaptoethanol. After saturation by shaking at room temperature, the aqueous phase is thoroughly removed and discarded.

Solution B:

35 75% v/v Isopropanol, 25% v/v 0.1M potassium acetate at pH 7.8.

Solution C:

75% v/v Isopropanol, 25% v/v 0.01M magnesium acetate.

(Note that other alcohols or similar water-miscible solvents, e.g. n-propanol, may be substituted for isopropanol in these solutions.)

3.2 Method.

All steps are preferably carried out in a single tube made of a suitable phenol resistant material, e.g. polyethylene.

(a) Removal of Protein: a 0.5cm x 0.5cm square of blood DNA impregnated paper is treated with 1 ml of solution A, for example for approximately 1.5 hours at 45°C (this temperature and time is not critical). The dirty solution A is then aspirated to waste and the paper square quickly washed three times with 0.25 ml of more solution A. Each wash need be only a few seconds long and is immediately aspirated to waste.

(b) Removal of phenol and addition of suitable ions: the paper in its tube from step (a) above, is rapidly washed in three lots of 1 ml of solution B. Washes are at room temperature and are simple additions followed by aspiration to waste. The paper is then washed for 20 minutes at room temperature with solution C. (This is to saturate the DNA on the paper with Magnesium ions and remove the last of the phenol.) The solution C is aspirated to waste and the paper is solvent-dried with one wash of pure isopropanol and then vacuum dried.

The final DNA-paper should be quite white without any obvious remnants of the red-brown colour of blood. It is now ready for use in a PCR reaction mix.

(c) Amplification of DNA on treated paper:

The treated DNA-paper as described above has been shown to be a suitable substrate for DNA polymerase chain reaction (PCR) amplification of DNA.

(i) Specimens

Extracted DNA: DNA from 10 ml of blood obtained from a male volunteer was extracted by standard protocols.

Treated DNA Filter Paper: Blood specimens from the same volunteer were applied directly to treated filter paper with subsequent treatment as described above. The paper was cut into about 1mm² pieces for use in PCR reactions.

(ii) Targets for Amplification.

Target No. 1: Region of exon 2 of the n-Ras proto-oncogene on chromosome 1. The primers used are:

R1: 5' TGA CTG AGT ACA AAC TGG TGG TG 3' and

R2: 5' CTC TAT GGT GGG ATC ATA TTC A 3'.

The amplified DNA fragment obtained with these primers is 110bp in size.

Target No.2: A male specific Y chromosome repeat sequence. The primers are:

007: 5' TGG GCT GGA ATG GAA AGG AAT GCA AAC 3' and

008: 5' TCC ATT CGA TTC CAT TTT TTT CGA GAA 3'.

The amplified DNA fragment obtained with these primers is 124 bp in size.

Target No.3: A male specific Y chromosome repeat sequence. The primers used are:

004: 5' GAA TGT ATT AGA ATG TAA TGA ACT TTA 3' and

006: 5' TTC CAT TCC ATT CCA TTC CTT TCC TTT 3'.

The amplified DNA fragment obtained with these primers is 250 bp in size.

(iii) PCR Protocol

Extracted DNA (1 μ g) or about 1mm² fragments of treated DNA filter paper were placed into 0.5ml Eppendorf tubes and made to 25 μ l in PCR reaction mixture consisting of:

67 mM Tris HCl (pH 8.8 @ 25°C)

16.6 mM ammonium sulfate

2 mM MgCl₂

0.01% (w/v) gelatin

0.1 mM deoxynucleotides (dATP, dTTP, dCTP, dGTP)

0.25 μ g of each primer (for respective target)

0.25 U of Taq DNA polymerase.

The mixture was overlaid with 25 μ l of light mineral oil and DNA amplification was performed by 30 cycles of amplification on a Perkin Elmer-Cetus "thermal cycler".

The first cycle consisted of:

DNA denaturation 6 min. @ 94°C

Primer-DNA annealing 1 min. @ 55°C

Taq DNA polymerase extension 1 min. @ 75°C

followed by 29 cycles as above except DNA denaturation was for 1 min @ 94°C and the extension time on the last cycle was 10 min @ 72°C before cooling of reaction mixture to 4°C.

After amplification, 10 μ l aliquots of PCR mixture were analysed by electrophoresis on 15% (w/v) polyacrylamide gels. Amplified target DNA was visualized by UV illumination of the ethidium bromide stained gel. An analysis of products from PCR of extracted DNA and treated DNA filter papers as shown in Figure 1, as follows:

Lanes 1-3: target No.1, lane 1: 1 μ g DNA, lane 2: 1mm² filter, lane 3: no DNA-control;

Lanes 4-5: target No.2, lane 4: 1 μ g DNA, lane 5: 1 mm² filter; Lane 6: no DNA control;

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Lanes 7-9: target No.3, lane 7: 1 μ g DNA, lane 8: 1 mm² filter, lane 9: no DNA control; lane S: DNA size markers (pUC19/HpaII digest).

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The results shown clearly demonstrate that the DNA has not been changed in any way as a result of its storage on filter paper in accordance with this invention, and that it can be used in situ as described herein.

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It will be readily apparent to those skilled in the art that the above solid-state steps for purification and amplification of blood DNA are particularly suited for performance under automated conditions.

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CLAIMS:

1. A solid medium for storage of DNA, including blood DNA, which comprises a solid matrix having a compound or composition which protects against degradation of DNA incorporated into or absorbed on the matrix.
2. A solid medium according to claim 1, wherein the solid matrix comprises a solid support selected from an absorbent cellulose-based paper or a micromesh of synthetic plastics material.
3. A solid medium according to claim 1, wherein the solid matrix includes a binder material and is in the form of a compressed tablet or pellet.
4. A solid medium according to claim 1, impregnated with or encased in a protective material.
5. A solid medium according to claim 4, wherein said protective material is a plastics material, such as polystyrene.
6. A solid medium for long term storage of purified DNA, which comprises a solid matrix having incorporated thereon or absorbed thereon a composition comprising uric acid or a urate salt, and a weak base.
7. A solid medium according to claim 6, wherein said weak base is tris-hydroxymethyl methane.
8. A solid medium for storage of blood DNA, which comprises a solid matrix having incorporated therein or absorbed thereon a composition comprising a weak base, a chelating agent, and an anionic surfactant or detergent.

9. A solid medium according to claim 8, further comprising uric acid or a urate salt.
10. A solid medium according to claim 8 or claim 9, wherein said composition has a pH of between 8.0 and 9.5.
11. A solid medium according to claim 8 or claim 9, wherein said weak base is tris-hydroxymethyl methane.
12. A solid medium according to claim 8 or claim 9, wherein said chelating agent is ethylene diamine tetracetic acid.
13. A solid medium according to claim 8 or claim 9, wherein said anionic surfactant or detergent is sodium dodecyl sulphate.
14. A solid medium for storage of blood DNA, which comprises an absorbent cellulose-based paper having a minimal loading, per sq. cm. of paper, of a composition comprising:
- (a) EDTA : 0.5 micromols (146.1 mg of free acid)
 - (b) Tris : 8 micromols (968.8 mg of free base)
 - (c) SDS : 1 mg, and optionally
 - (d) uric acid : 2 micromols (336.24 mg of acid).
15. A method for the storage of DNA, including blood DNA, which comprises applying the DNA to a solid medium which comprises a solid matrix having a compound or composition which protects against degradation of DNA incorporated in or absorbed on the matrix.
16. A method according to claim 15, which comprises applying the DNA to a solid medium having incorporated therein or absorbed thereon a composition comprising uric acid or a urate salt, and a weak base.

17. A method according to claim 15, which comprises applying blood DNA to a solid medium having incorporated therein or absorbed thereon a composition comprising a weak base, a chelating agent, and an anionic surfactant or detergent, and optionally uric acid or a urate salt.

18. A method according to claim 15, further comprising the step of subsequent removal of the DNA from the solid medium.

19. A method according to claim 15, further comprising in situ treatment of the stored DNA.

20. A method according to claim 19, wherein said treatment includes the step of removal of protein from the stored DNA.

21. A method according to claim 19 or claim 20, wherein said in situ treatment includes the step of DNA sequence amplification by the polymerase chain reaction technique.

22. A method according to claim 15, further comprising the step of impregnating said solid medium with or encasing said solid medium in a protective material.

23. A method according to claim 22, wherein said protective material is a plastics material, such as polystyrene.

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*Fig.1.***SUBSTITUTE SHEET**

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 89/00430

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6				
According to International Patent Classification (IPC) or to both National Classification and IPC				
Int. Cl. ⁴ C07B 63/00, C12M 1/16, C12Q 1/68				
II. FIELDS SEARCHED				
Minimum Documentation Searched 7				
Classification System	Classification Symbols			
IPC ⁴	C07B 63/00, 63/04, C07H 21/04, C12M 1/16, C12N 1/00, 11/12, C12Q 1/68			
	C.A., WPI, WPIL, Keywords: DNA; STORAGE; PRESERVATION			
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8				
AU : IPC as above				
III. DOCUMENTS CONSIDERED TO BE RELEVANT 9				
Category*	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13		
A	EP,A, 0261955 (E.I. DU PONT DE NEMOURS AND COMPANY) 30 March 1988 (30.03.88)			
A	AU,B, 30256/84 (MOLECULAR DIAGNOSTICS INC) 10 January 1985 (10.01.85)			
A	EP,A, 0221308 (MILES LABORATORIES, INC.) 13 May 1987 (13.05.87)			
<p>* Special categories of cited documents: 10</p> <table style="width: 100%;"> <tr> <td style="width: 50%; vertical-align: top;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 50%; vertical-align: top;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p> </td> </tr> </table>			<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>
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IV. CERTIFICATION				
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report			
21 December 1989 (21.12.89)	4-1-90			
International Searching Authority	Signature of Authorized Officer			
Australian Patent Office	JOHN ASHMAN <i>John Ashman</i>			

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[illegible]

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

- VI. [◇] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

1. [X] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

4. [X] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

[☒] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 89/00430

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Members			
EP	261955	JP 63098400			
EP	130523	AU 30256/84	AU 563558	CA 1215703	
		DE 3471689	ES 534025	ES 8607387	
		IL 72278	JP 60036496	US 4542102	
		US 4713326			
EP	221308	DE 3666707	JP 62081565	US 4806546	

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